AUTORADIOGRAPHIC LOCALIZATION OF CARBACHOL-INDUCED SECOND MESSENGER RESPONSE IN THE RAT SPINAL CORD FOLLOWING INFLAMMATION

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This study examined central mechanisms of persistent pain using an autoradiographic technique to localize phosphoinositide hydrolysis (PI) in the rat spinal cord dorsal horn. The lateral half of laminae I-II showed the highest levels of baseline PI turnover and carbachol-stimulated PI turnover in normal animals as well as after inflammation. Inflammation resulted in increased baseline PI turnover in this region of the ipsilateral (76%) and contralateral (65%) dorsal horns. Carbachol increased PI turnover in this region in normal rats (55%) and following inflammation (ipsilateral: 46%, contralateral: 45%). The absolute magnitudes of these increases were 1.85, 2.71, and 2.51 nCi/mg, respectively.

The results of this study demonstrate the involvement of PI turnover in neural mechanisms of persistent pain, and provide evidence for the involvement of cholinergic systems in this process. Because spinal cholinergic systems have been reported to be anti-nociceptive, the present results appear to reflect an upregulation of anti-nociceptive activity in response to inflammation. Thus, the spinal cholinergic system may be a regulatory site within the anti-nociceptive pathway, and may provide an attractive target for the development of new therapeutic agents.

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INTRODUCTION

The focus of this study is to examine changes in dorsal horn neurons during peripheral inflammation. These changes include increased responsiveness to suprathreshold stimuli, decreased response thresholds, receptive field expansion, and sensitivity to non-noxious stimuli. These changes underlie hyperalgesia and allodynia associated with persistent pain. Therefore, it is critical to understand what mediates these changes in addition to classifying them. The long lasting effects of hyperalgesia may be mediated by changes in second messenger systems in neurons associated with pain signaling. Phosphoinositide (PI) hydrolysis is a second messenger system that mediates the effects of many neurotransmitters acting at G-protein coupled receptors, including acetylcholine acting on muscarinic receptors. Neurotransmitter-stimulated PI turnover can be assessed in spinal cord slices using a radiolabeled precursor to an intermediate of the PI cycle that becomes incorporated into the cell membrane where it can be quantified. In addition, the distribution of the PI turnover within the spinal cord can be established and compared to areas within the dorsal horn known to receive nociceptive input. Elucidating pain-related changes in this pathway may provide new therapeutic targets for pharmacological agents in the treatment of persistent pain.

Background and significance

Persistent pain can increase the excitability of neurons in the pain pathway, especially in the dorsal horn of the spinal cord. This increase in excitability is termed sensitization. The changes underlying sensitization have most likely evolved to serve a protective function to avoid further damage to injured tissue. Defining these changes should aid in developing new therapeutic approaches in the treatment of pain. The neural mechanisms underlying sensitization have been studied employing techniques to measure neurotransmitter levels, receptor densities, and electrophysiological changes. An additional approach, used in this study, is to examine a second messenger system associated with cholinergic neurons in the dorsal horn of the spinal cord. A benefit of studying second messenger systems is that functional changes in the neurotransmitter/receptor system can be assessed. This approach may provide evidence of regulation within the pain pathway. This study examined functional changes in the pain pathway as revealed by the phosphoinositide second messenger response to a specific cholinergic agonist.

Models of persistent pain

Models of persistent pain can be classified as *neuropathic* or *inflammatory*. Neuropathic pain refers to pain caused by damage to the nervous system. Neuropathic pain in animal models is typically established by loose ligation of a peripheral nerve, nerve crush, partial nerve transection, or complete nerve transection. Neuropathic models

exhibit varying degrees of spontaneous pain behaviors, increased sensitivity of dorsal horn neurons, and hyperalgesia (Bennett, 1993).

Inflammatory models of pain involve cutaneous tissue damage, usually provoked by chemical irritants. These chemical irritants include mustard oil, carrageenan, formalin, and complete Freund's adjuvant (CFA). Mustard oil is a topical agent and is therefore not suitable for studies of persistent pain. Carrageenan, formalin, and CFA are administered by subcutaneous injection, often into the plantar surface of the hind paw. These three agents differ in the time course of the inflammatory state that is produced. Formalin produces its effects immediately, carrageenan's effects peak at approximately 3 hours, and the effects of CFA peak over the course of 2-3 days (Honore et al., 1999).

CFA was the model of choice for the present study due to its time course of activity. Since CFA is considered a long-term model, it provides a good opportunity to examine long-term central changes associated with PI turnover. CFA has been widely used in the study of persistent pain and its effects are well characterized. Although neuropathic models also cause long-term changes, some of these include reorientation of the terminals of some primary afferent fibers to different dorsal horn laminae and reduction or degeneration of inhibitory interneurons (primarily in laminae I and II) in the dorsal horn (Nakamura and Meyers, 2000; Ibuki et al., 1997; Mao et al., 1997; Nachemson and Bennet, 1993; Sugimoto et al., 1989). Since the present study localized PI turnover resulting from activation of synapses formed by inhibitory interneurons these are obviously undesirable effects.

CNS changes associated with persistent pain

Evidence for central changes associated with persistent pain comes from studies of zones of hyperalgesia. Inflammatory pain is associated with two principle zones of hyperalgesia in the periphery (Hardy et al., 1950). The first is a zone of primary hyperalgesia, and is comprised of the region of tissue damage itself. Primary hyperalgesia is characterized by spontaneous pain and increased sensitivity to heat and mechanical stimuli.

There is a large body of evidence documenting the sensitization of primary afferent neurons (e.g., Beitel and Dubner, 1976; Perl et al., 1976; Campbell and Meyer, 1983; Koltzenburg et al., 1992), that reveals a peripheral mechanism underlying the primary hyperalgesia. A zone of secondary hyperalgesia involves undamaged tissue surrounding the site of injury. Dorsal horn neurons in the representation of the zone of secondary hyperalgesia display increased sensitivity to mechanical but not heat stimuli. Secondary hyperalgesia appears to involve a central sensitization. Initial evidence for central sensitization was obtained by Thalhammer and LaMotte (1982), who found that cutaneous hyperalgesia spreads well beyond the area of nociceptor sensitization. In addition, hyperalgesia that develops after capsaicin injection remains even after anesthetizing the region of injection, but if the region is anesthetized prior to capsaicin injection hyperalgesia does not develop (LaMotte et al., 1991). Furthermore, capsaicin induced hyperalgesia can be prevented by a proximal block of the peripheral nerve that innervates the area corresponding to the injection. This clearly demonstrates the

requirement for nociceptive information to reach the spinal cord to establish secondary hyperalgesia.

A variety of neurotransmitters and neuromodulators related to nociceptive processing in the dorsal horn have been identified and display varying degrees of colocalization and corelease (reviewed by Millan, 1999). At least three of these appear to be essential for the establishment of sensitization: glutamate, substance P, and nitric oxide. Both NMDA and substance P are necessary for induction of hyperalgesia, whereas NMDA is also necessary for the maintenance of hyperalgesia. Nitric oxide appears to mediate the effects of NMDA in producing hyperalgesia.

The majority of work in the pain field has concentrated on the nociceptive component of the pain system, yet there is also a critical anti-nociceptive component. It should be evident that a balance must exist between the nociceptive and anti-nociceptive components for normal pain transmission. Changes in this balance could lead to detrimental effects on pain perception such as the sensitization described above. Therefore it is important to understand the role of the anti-nociceptive component in persistent pain.

Anti-nociception

The pathways involved in anti-nociception are beginning to be resolved, but the nature of their regulation in response to persistent pain is not known. It is possible that anti-nociceptive pathways are upregulated in response to persistent pain. It is also possible, however, that persistent pain produces a loss of inhibitory tone, which in turn

produces sensitization. A better understanding of how these pathways are regulated should aid in deriving alternative therapeutic approaches in the treatment of pain.

The existence of critical factors involved in endogenous anti-nociception remains unclear. This is partially due to the complex nature of the anti-nociceptive pathway. The anti-nociceptive pathway involves both descending and intrinsic spinal pathways, and involves at least twenty-five different neurotransmitters. The bulk of present research focuses on six different neurotransmitters: acetylcholine (ACh), opioids, norepinephrine, serotonin, gamma aminobutyric acid (GABA), and nitric oxide. It appears that norepinephrine and serotonin are the primary neurotransmitters that are released by descending neurons of the anti-nociceptive pathway. At least a portion of the descending anti-nociceptive effects is mediated by ACh. ACh also participates in intrinsic anti-nociceptive pathways, along with the opioids, nitric oxide, and GABA.

Descending Anti-nociceptive Systems

Stimulation of bulbospinal neurons in the nucleus raphe magnus (NRM) decreases responsiveness to noxious stimuli (Oleson et al., 1978; Oliveras et al., 1979; Oliveras et al., 1975; Proudfit et al., 1975) by inhibiting dorsal horn neurons that are activated by noxious stimuli (Basbaum et al., 1976; Duggan et al., 1979; Fields et al., 1977; Guilbaud et al., 1977). Several lines of evidence suggest that adrenergic and serotonergic neurons are involved in this effect. Serotonin and norepinephrine are released into spinal cord superfusates in response to electrical stimulation of the NRM (Hammond et al., 1985). Intrathecal injection of either serotonergic or noradrenergic antagonists blocks the anti-

nociception produced by electrical stimulation of the NRM (Hammond and Yaksh, 1984).

Intrinsic Anti-nociceptive Systems

Intrinsic neurons of the spinal cord reside in the cord and do not project to supraspinal locations. These neurons can be classified as intersegmental (propriospinal), interlaminar intrasegmental (interneurons), and intralaminar intrasegmental (interneurons) (Willis and Coggeshal, 1991). The intrinsic neurons of the dorsal horn are also known to exhibit varying degrees of neurotransmitter colocalization. For example: GABA-glycine, GABA-enkephalin, enkephalin-SP, and GABA-nitric oxide synthase are colocalized (Todd and Sullivan, 1990; Riberio-da-Silva and Coimbra, 1980; Todd et al., 1992; Senba et al., 1988 Tashiro et al., 1987; Ribeiro-da-Silva et al., 1991; Laing et al., 1994). It appears that anti-nociceptive effects are at least partially mediated by intrinsic neurons.

ACh in the Spinal Cord

It is generally thought that ACh-induced anti-nociceptive effects arise from intrinsic neurons since evidence shows a lack of cholinergic projection neurons (Sherriff et al., 1991; Eisenach, 1999; Barber et al., 1984). ACh is also known to be colocalized with GABA and nitric oxide synthase (Spike et al., 1993; Laing et al., 1994; Todd et al., 1991; Kluchova et al., 2000). Muscarinic receptors are concentrated primarily in lamina

II with some in lamina III, whereas nicotinic receptor concentrations have been demonstrated in laminae I, III, and IV (Coggeshall and Carlton, 1997).

Acetylcholine and Anti-nociception

There is considerable evidence to support the finding that ACh is anti-nociceptive in the dorsal horn. Spinal cord ACh content is significantly increased following formalin injection, and intrathecal injections of muscarinic antagonists inhibit the second phase (prolonged phase of tonic pain) of the nociceptive response (Honda et al., 2000). Intrathecal administration of carbachol (a muscarinic agonist) or acetylcholinesterase inhibitors produces anti-nociception (Taylor et al., 1982; Gillberg et al., 1989; Abram and O'Connor, 1995; Bouaziz et al., 1995) that can be inhibited by the muscarinic antagonist atropine (Zhuo and Gebhart, 1991; Naguib and Yaksh, 1994). Cholinergic anti-nociception appears to involve both muscarinic and nicotinic receptors since both produce analgesia when administered intrathecally, and the analgesia can be reversed by addition of specific nicotinic and muscarinic antagonists (Yaksh et al., 1985; Rao et al., 1996; Lawand et al., 1999; Marubio et al., 1999; Pan et al., 1999). However, most of the anti-nociceptive effects of acetylcholine appear to be mediated through muscarinic receptors: intrathecal muscarinic antagonists nearly abolish anti-nociceptive effects whereas nicotinic antagonists only attenuate about 40% of the effect (Pan et al., 1999). The effects of nicotinic agonists are further complicated depending on their route of administration. If administered intrathecally they demonstrate nociceptive and anti-

nociceptive properties (depending on concentration), whereas, if given systemically they produce anti-nociceptive effects (Rueter et al., 2000).

Mechanisms of ACh Anti-nociception

Acetylcholine likely exerts its anti-nociceptive actions through both pre- and postsynaptic mechanisms including activation of other inhibitory interneurons (Fig. 1). Evidence obtained by Naguib and Yaksh (1997) suggest that M1 and/or M3 receptor subtypes mediate cholinergic anti-nociception.

The precise role of ACh in anti-nociception remains unclear, perhaps due to considerable interaction between ACh and other neurotransmitter systems. Some of these interactions are described below. Some of ACh's anti-nociceptive actions are mediated through reductions in spinal levels of SP. This was demonstrated by intrathecal administration of carbachol following the application of radiant heat. Carbachol increased tail flick latencies and reduced spinal levels of SP in the dorsal horn by 30%, and both of these effects were inhibited by atropine (Smith et al., 1989).

At least a portion of cholinergic interneuron activation appears to be mediated by descending serotonergic and adrenergic pathways. There is strong evidence for cholinergic activation by the adrenergic pathway. ACh release in the spinal cord is stimulated by intrathecal administration of clonidine (De Kock et al., 1997; Klimscha et al., 1997). Intrathecal administration of an acetylcholinesterase inhibitor plus an adrenergic agonist (clonidine) provides a more profound anti-nociception than clonidine alone (Gordh et al., 1989). Further evidence for ACh interaction with descending

adrenergic and serotonergic pathways comes from studies in which spinal anti-nociception produced by carbachol was inhibited by depletion of descending adrenergic neurons (Gillberg et al., 1989) or by addition of adrenergic or serotonergic antagonists (Zhuo and Gebhart, 1990). These studies provide evidence for descending anti-nociceptive pathways whose effects are mediated by ACh within the spinal cord.

Cholinergic-mediated anti-nociception also involves nitric oxide. Intrathecal clonidine causes ACh release from the spinal cord (De Kock et al., 1997; Klimscha et al., 1997), which in turn stimulates nitric oxide synthesis (Xu et al., 1997). Activation of muscarinic as well as nicotinic receptors appears to stimulate nitric oxide release in the spinal cord (Xu et al., 2000). Anti-nociception resulting from supraspinal (Iwamoto and Marion, 1994b) or intrathecal (Iwamoto and Marion, 1994a) administration of cholinergic agonists is antagonized by nitric oxide synthase inhibitors. Nitric oxide synthase inhibitors also block the enhancement of anti-nociception produced by intrathecal administration of clonidine or neostigmine (Xu et al., 1996).

ACh also appears to play a role in opioid mediated analgesia. A study by Kang et al. (1997) suggests that analgesia produced by muscarinic receptors at the spinal level might be mediated by endogenous opioids and ATP-sensitive potassium channels in a cascade form since (1) carbachol anti-nociception could be antagonized by atropine; (2) carbachol anti-nociception could be blocked by glibenclamide (blocker of ATP sensitive potassium channels) or naloxone (opiate antagonist); (3) morphine anti-nociception could be blocked by glibenclamide but not by atropine. These data suggest that carbachol

activates opioidergic neurons which in turn open ATP sensitive potassium channels postsynaptically.

Additional evidence for cholinergic interactions in anti-nociceptive processing comes from a recent study of GABAergic interneurons in the dorsal horn of the rat spinal cord (Baba et al., 1998). GABA release in the substantia gelatinosa is facilitated by the cholinergic agonist carbachol. Carbachol and neostigmine increase TTX-sensitive GABAergic IPSCs in the majority of substantia gelatinosa neurons. Carbachol, but not neostigmine, increases the quantal release of GABA from presynaptic terminals. This facilitatory effect of carbachol was antagonized by atropine.

The involvement of spinal cholinergic systems with multiple anti-nociceptive neurotransmitters suggests that they may play a key role in anti-nociception. Moreover, the spinal cholinergic system might present an alternative treatment opportunity, with fewer of the harmful side effects that are seen with opioid treatments. Some muscarinic receptors exert their effects by stimulating PI turnover. The regulation of PI turnover provides a good target for studying pain processing because it reflects changes in functional aspects of cell processing.

Phosphatidylinositol Turnover in the Spinal Cord

Studies of (PI) hydrolysis utilizing anion exchange chromatography of radiolabeled inositol suggest that there is endogenous PI turnover in spinal cord (Prat et al., 1993; Parsons et al., 1995) and brain (Bymaster et al., 1998). These studies reported stimulated PI hydrolysis as a percent increase over baseline. Using cross-chopped slices

Hassessian et al (1992) demonstrated basal PI turnover that could be antagonized by the anesthetic pentobarbital but not urethane. Because these studies were done on cross-shopped slices, the distribution of PI turnover within the spinal cord could not be ascertained. One aim of this study was to measure the magnitude and location of endogenous PI turnover within the dorsal horn to allow for comparisons between endogenous and stimulated PI turnover in normal animals and in response to inflammation.

The utility of analyzing neurotransmitter-stimulated PI turnover lies in the ability to localize the functional effects of neurotransmitter actions, and to determine if these change with persistent pain. Many previous studies have used receptor binding to gauge changes in neurotransmitter effectiveness. For example, it has been demonstrated that hindpaw inflammation and chronic constriction injury produce an increase in the affinity of substance P receptors in the dorsal horn (Stucky et al., 1993; Aanonsen et al., 1992). These results are supported by evidence that inflammation increases SP receptor immunoreactivity (Abbadie et al., 1996; Kar et al., 1993; Schafer et al., 1993; McCarson and Krause, 1994). However, changes in receptor binding do not necessarily reflect the function of receptors. For example, increased expression of NK 1 receptors in cultured spinal cord neurons does not result in increased generation of PI (Abrahams et al., 1999). This is further supported by the work of Holland et al. (1993), showing that SP receptors and G proteins can uncouple with repeated stimulation. Recent evidence also indicates that changes in PI signaling can exist without changes in 5-HT receptor number or affinity (Toscano et al., 1999).

Therefore, in order to understand the role of a neurotransmitter/receptor system in pain signaling it is useful to also investigate receptor function as seen in second messenger responses. If changes in PI turnover are detected, changes in receptor numbers or affinities can then be studied in order to begin to localize the site of regulation.

Binding of ACh to a PI linked receptor activates a G protein (G_q), which activates phospholipase C to cleave PIP_2 into two second messengers, diacylglyceraol (DAG) and IP_3. DAG activates protein kinase C (resulting in protein phosphorylation), and IP_3 releases Ca^{++} from intracellular stores (Fig. 2).

Measurement of PI turnover is usually done on crosschopped slices (typically 350 x 350 µm). The slices are incubated with radiolabeled myo-inositol in the presence of lithium in Krebs buffer solution. Receptor activation resulting from the addition of a neurotransmitter agonist causes the accumulation of labeled inositol phosphates, which are analyzed by anion exchange chromatography. The use of [^{3}H]cytidine as a precursor was developed by Godfrey (1989) for biochemical analyses, and was recognized by Solomon Snyder as being suitable for autoradiography because the intermediate [^{3}H]CDP-DAG is membrane bound and does not wash out during subsequent treatments (Hwang et al., 1990). The obvious advantage of the autoradiographic method is that it allows the investigator to localize the response histologically.

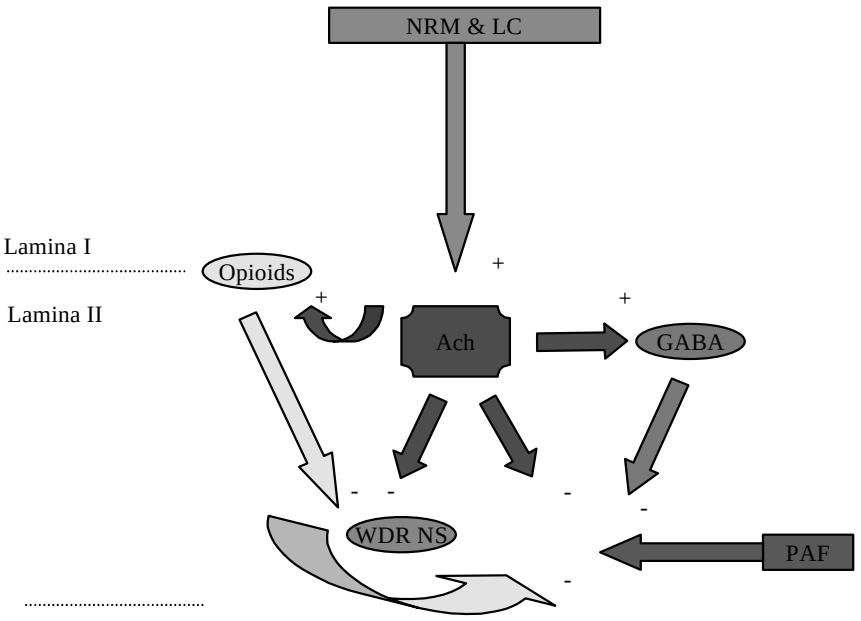

Figure 1. Diagram showing the putative relationship between descending and intrinsic components of the anti-nociceptive systems in the dorsal horn. NRM=nucleus raphe magnus, LC=locus coeruleus, PAF=primary afferent fiber, WDR=wide dynamic range, NS=nociceptive specific.

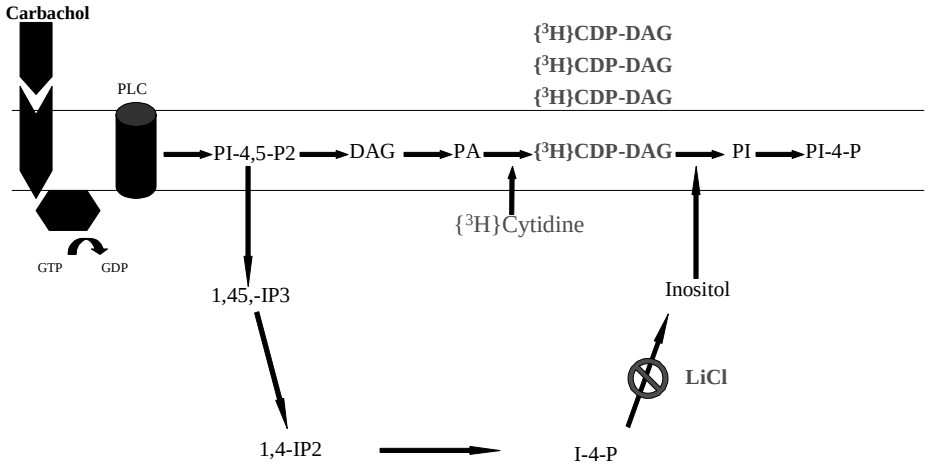

Figure 2. Diagram of the method used to assess PI turnover in spinal cord slices. The sites where LiCl acts and where the radiolabeled cytidine is incorporated are indicated (Hwang et al., 1990).

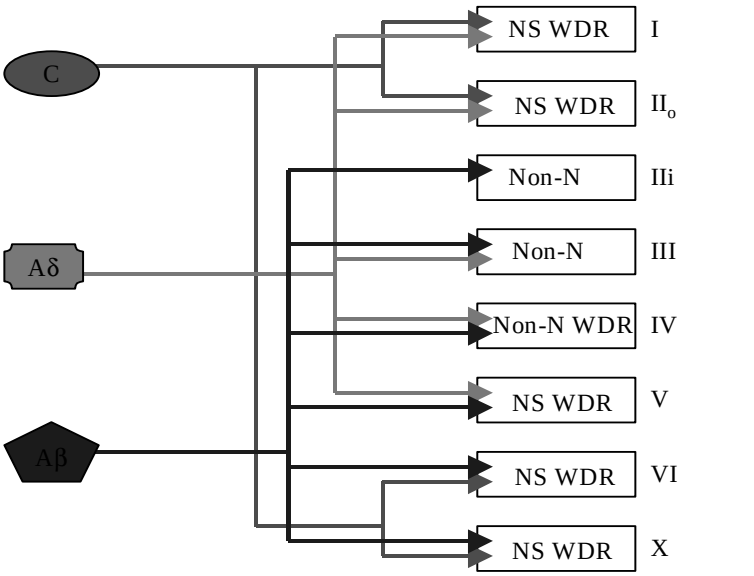

Figure 3. Diagram of dorsal horn innervation by primary afferent fibers illustrating laminar termination patterns as well as the type of neuron targeted. NS=nociceptive specific, WDR=wide dynamic range, LTM=low threshold mechanoreceptor.

CHAPTER 2

EXPERIMENTAL METHODS AND PROCEDURES

Studies utilizing the technique used in this study have localized PI turnover in intact brain slices (Hwang et al., 1990; Robinson et al., 1993; Bevilacqua, 1995). However, no one has focused on the spinal cord using this method. Previous studies have provided evidence that levels of certain receptors are significantly affected by inflammation (Stucky et al., 1993; Aanonsen et al., 1992; Abbadie et al., 1996; Kar et al., 1993; Schafer et al., 1993; McCarson and Krause, 1994). ACh receptors however, have not been studied with respect to inflammatory effects. Measurement of PI turnover allowed us to assess the impact of persistent pain on the function of muscarinic ACh receptors.

Subjects and Anesthesia

Subjects were P30-P35 male Long-Evans hooded rats. Ketamine (100 mg/kg i.m.) was used as the anesthetic for the purpose of hindpaw inflammation. Subsequent surgical removal of the spinal cord was performed two days later under deep anesthesia via urethane (2 g/kg i.p.). Barbiturates were not used due to evidence presented by Hassessian et al. (1992) that they significantly reduce basal PI turnover.

Inflammation

Hindpaw inflammation was achieved by injecting complete Freund's adjuvant (CFA). The CFA was emulsified 1:1 with phosphate buffer for a total volume of 150µl, which was then injected into the plantar plexus of the hindpaw. Control animals were not injected with vehicle because such injections would produce painful stimulation.

Surgery and Slice Preparation

Two days after CFA injection each animal was deeply anesthetized and the dorsal half of the vertebral column and the dura mater were removed. India ink was used to mark the L4 region ipsilateral to the inflamed hindpaw. The L4 region of the cord was then removed and placed into cold (approximately 4°C) Krebs buffer (114 mM NaCl, 4.57 mM KCl, 2.44 mM $CaCl_2$, 1.14 mM KH_2PO4, 1.2 mM $MgSO_4$, 24.7 mM $NaHCO_3$, 10 mM glucose, 0.1 mM ascorbic acid, pH 7.4). The cord was then transferred to the stage of a McIlwain tissue chopper and cut into 400 µm slices. The slices were transferred to a dish of Krebs buffer (room temperature) where the L4 segment slices were carefully separated with brushes.

Recovery

Slices were placed on a 3 µm Millicell-PC tissue culture platform (Millipore, Marlboro MA) and submersed in 5 ml Krebs buffer at room temperature. The recovery period began with 30 min in low calcium/high magnesium (0.5 mM Ca^{++}, 10 mM Mg^{++}) Krebs buffer, followed by an additional 30 min period in the standard Krebs buffer. The

buffer was changed every 15 min during the recovery period, and all solutions were continuously aerated with 95% O_2 / 5% CO_2.

Autoradiographic Method for Assessing PI Turnover.

The methods were based on those of Hwang et al. (1990) with modifications (Robinson et al. 1993). Following the recovery period, each Millicell platform was transferred to a tissue culture plate (Fig. 4) containing inhibitor solution (500 µl Krebs buffer with 200 µl nucleic acid synthesis inhibitor (0.9 µg actinomycin D and 50 mM hydroxyurea in Krebs-carbonate buffer) for 10 min. The tissue culture plate was kept in a water bath at 37°C with constant aeration of 95% O_2/5% CO_2. After 10 min 200 µl of [^{3}H]cytidine (21.5Ci/mmol, Sigma; lyophilized in buffer) was added for a final concentration of 10µCi/ml. After an additional 30 min, 50µl of LiCl (5mM) was added to block recycling of the intermediates. Five minutes after adding lithium, half of the sections from each animal were incubated with the muscarinic agonist carbachol (1mM) for 45 min and the other half were incubated without agonist. Sections from control animals (non-inflamed) were also divided into agonist and no agonist incubations.

Sectioning the Slice

After the incubation, slices were transferred to an embedding mold. Embedding compound (O.C.T., Miles) was poured over the slice; the mold was covered and placed into an ultra cold freezer (-80°C). The slice was sectioned at 40µm in a cryostat and thaw-mounted onto gelatinized slides.

Removal of [^{3}H]cytidine Incorporated into Nucleic Acids

Slide-mounted sections were washed for 5 min in a nuclease rinse (50mM Tris-Hcl, 2mM EDTA, 10mM LiCl, 3% polyethylene glycol, 20µg/ml RNase A, and 20µg/ml DNase), rinsed in dH_2O and air-dried.

Autoradiography and Histology

Following the nuclease rinse the sections were exposed to tritium sensitive film (^{3}H-Hyperfilm, Amersham) along with tritium standards (ART 123A, American Radiolabeled Chemicals). The exposure period was four weeks. The film was then developed with Kodak D19 and further processed according to the manufacturer's directions. Spinal cord sections were stained for cytochrome oxidase activity since the majority of Nissl substance was removed by the nuclease rinse prior to exposure to film.

Data Analysis

The data were collected using a computerized video-based densitometry system (MCID M-4, Imaging Research, St. Catherines, Ontario). Film densities were calibrated with respect to a best-fit curve based on the tritium standards. The digitized image was visualized on a color monitor where it was then overlayed with the image of the cytochrome oxidase-stained section. Using the cytochrome oxidase image, Rexed's laminae 1-6 were outlined and the data were collected from the autoradiograph (Fig. 5) via redirected sampling from the outlined laminae.

For data sampling both ipsilateral and contralateral sides for each section were further divided into a medial half and a lateral half. Data were then collected from

laminae I-II, III-IV, and V-VI regions for each half. This gave readings from twelve locations on each section (IpsiMed I-II, IpsiLat I-II, ContraMed I-II, ContraLat I-II, IpsiMed III-IV, IpsiLat III-IV, ContraMed III-IV, ContraLat III-IV, IpsiMed V-VI, IpsiLat V-VI, ContraMed V-VI, and ContraLat V-VI).

Statistical Analysis

For each animal, medians of laminar density readings were calculated for analysis due to the small sample size. Statistical analysis was performed using Jandel SigmaStat software. Except where noted, data were analyzed using a two-way analysis of variance, with treatment or side (ipsilateral, contralateral) of spinal cord as one factor and laminar location as the other factor. Tukey's test was used for post-hoc analyses except where noted. Significance for all analyses was set at $p<0.05$.

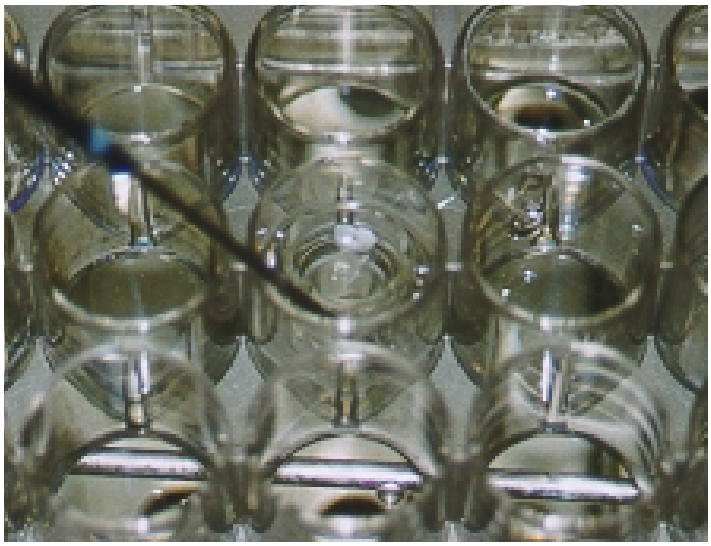

Figure 4. Tissue culture plate aerated in 37°C water bath containing a Millicell well with a spinal cord section immersed in buffer.

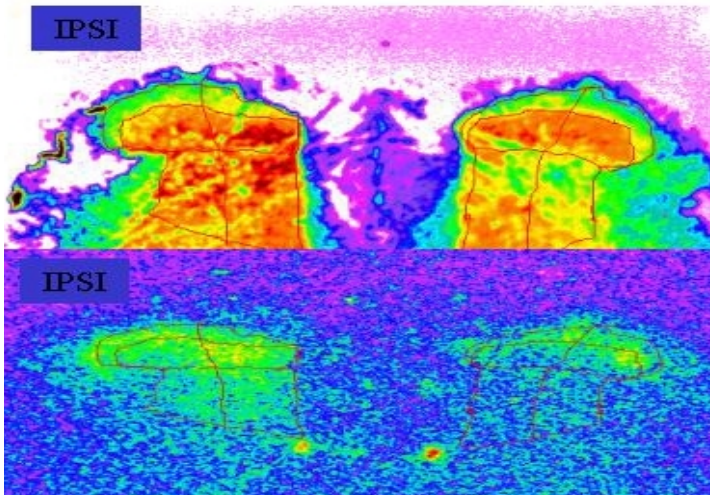

Figure 5. Digitized images from image analysis system showing the cytochrome oxidase stained image (upper) with laminar boundaries depicted. Bottom image shows the film image with the corresponding laminar boundaries from the upper image.

CHAPTER3

Results

Cytochrome oxidase activity was restricted to the spinal gray matter with little or no staining in the white matter (Fig. 6). The lightest area of staining was in laminae I and II of the superficial dorsal horn. More intense staining was found in the deep dorsal horn and the ventral horn. Stain intensity was uniform across the mediolateral aspect of the dorsal horn. Intense staining was also observed around the anterior median artery.

Baseline PI turnover in spinal cord of non-inflamed rats

Measures of PI turnover in control animals were based on combined readings from both sides of the spinal cord. Baseline (non-stimulated) PI turnover in non-inflamed rats was confined to the spinal gray matter. Within the gray matter, labeling was concentrated in the dorsal horns and around the central canal. The labeling was highest in the superficial laminae of the dorsal horn and declined with increasing depth. Across the dorsal horn, lateral regions were more densely labeled than medial regions. This general pattern of labeling was observed in all experimental groups (Fig. 7). As a result of this pattern of labeling, all statistical tests revealed a significant effect of laminar location. Therefore in the following sections only significant interactions between treatment and location, or significant effects of treatment, will be described in detail. Reduced synaptic activity (incubation in low Ca^{++}/ high Mg^{++} buffer) resulted in significantly lower levels of PI turnover in the dorsal horn ($F_{1,72}$=21.65, $p<0.001$, Figure 8). On average, labeling was reduced by 35%, with a range of 24-45% across laminar locations.

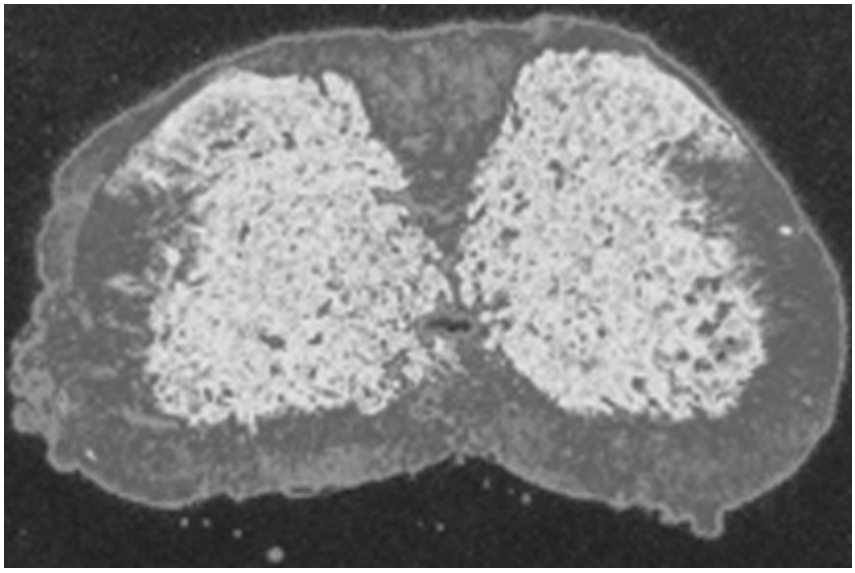

Figure 6. Digitized image of section stained for cytochrome oxidase activity. The lightest area of staining in the gray matter was found in Laminae I-II. The darkest staining was found in the deep dorsal horn and ventral horn.

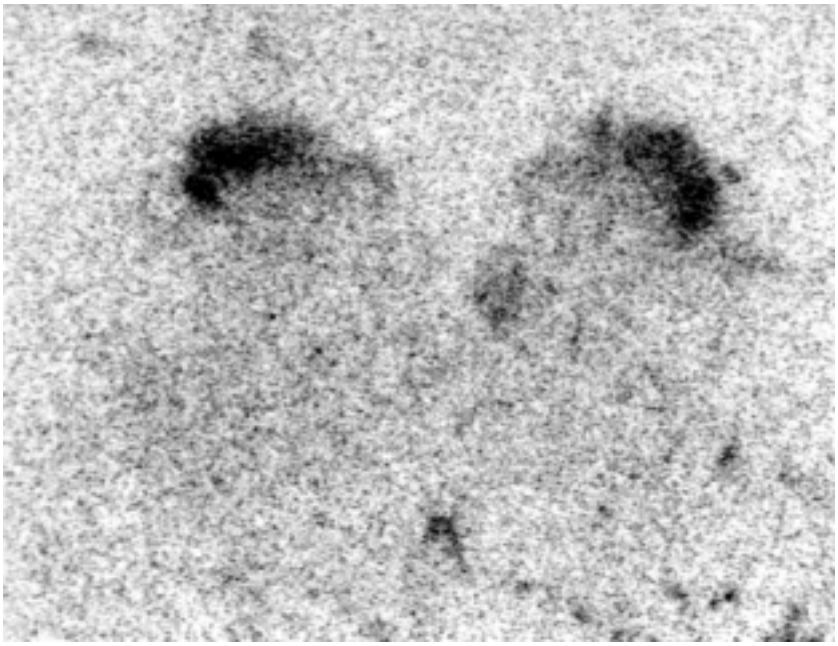

Figure 7. Digitized image of autoradiograph showing PI labeling in the dorsal horn of the spinal cord. Labeling is concentrated in the lateral portion of the superficial dorsal horn.

Carbachol-stimulated PI turnover in non-inflamed rats

The addition of carbachol to the incubation solution resulted in significantly higher levels of PI turnover in the dorsal horn compared to sections incubated without carbachol ($F_{1,78}$=19.23, $p<0.001$, Figure B). The average increase was 36%, with a range of 18-55%. The largest increase was in the lateral half of laminae I-II (Figure 9).

The effects of inflammation on baseline PI turnover

The general pattern of PI labeling in the dorsal horn of inflamed animals was similar to that seen in control animals. Statistical comparison of the ipsilateral and contralateral sides of the spinal cords from these animals revealed only a significant effect of laminar location ($F_{5,108}$=33.78, $p<0.001$, Figure 10). Post-hoc analyses (Tukey test) revealed that labeling in the lateral half of laminae I-II was significantly higher than in the other laminar locations. The next highest labeling was found in the medial half of laminae I-II, followed by the lateral half of laminae III-IV. PI turnover in sections from inflamed animals was higher than in sections from non-inflamed animals. Comparing ipsilateral and contralateral sides to sections from non-inflamed animals, there were significant main effects of location ($F_{5,162}$=36.29, $p<0.001$) and treatment ($F_{2,162}$=16.71, $p<0.001$, Fig 11). Post hoc analyses revealed that both sides of spinal cords from inflamed rats showed increased PI turnover (average increases of 35% in the ipsilateral side and 31% in the contralateral side).

Incubation in low calcium buffer to reduce synaptic activity resulted in reduced PI labeling. Comparing ipsilateral and contralateral sides to sections from non-inflamed animals, there were significant main effects of location ($F_{5,54}$=8.87, $p<0.001$) and

treatment ($F_{2,54}$=7.59, p<0.001, Fig 12). Post hoc analyses revealed that both sides of spinal cords from inflamed rats showed reduced PI turnover (average reductions of 26% in the ipsilateral side and 17% in the contralateral side).

The effects of inflammation on carbachol stimulated PI turnover

Levels of PI turnover in sections from inflamed animals were increased by the addition of carbachol to the incubation solution. A two-way analysis of variance revealed a significant interaction between treatment and laminar location ($F_{11,192}$=2.51, p<0.006, Fig. 13). On both the ipsilateral and contralateral sides, the lateral half of laminae I-IV and the medial half of laminae I-II showed significant increases, ranging from 33 to 46%.

Compared to sections from non-inflamed animals, carbachol stimulated higher levels of PI turnover in sections from inflamed animals. A two-way analysis of variance revealed significant main effects of laminar location ($F_{5,108}$=43.82, p<0.001) and treatment (non-inflamed vs. ipsilateral inflamed vs. contralateral inflamed; $F_{2,108}$=8.95, p<0.001, Fig. 14). On average, the ipsilateral side of spinal cords from inflamed animals showed a 32% increase in PI turnover, and the contralateral side showed a 25% increase. Within laminar positions, the increases ranged from 9-65% on the ipsilateral side and from 3-55% on the contralateral side.

In all of the experiments, the lateral half of laminae I-II showed the densest labeling. This was true for baseline PI turnover and carbachol-stimulated PI turnover in non-inflamed animals as well as after inflammation. Without carbachol stimulation, PI turnover in this laminar location following inflammation was 76% (ipsilateral) and 65% (contralateral) higher than in non-inflamed (3.34, 5.87 and 5.51 nCi/mg, respectively).

With carbachol stimulation, PI turnover increased in this location in each of the conditions: non-inflamed (55%; 3.34 to 5.19 nCi/mg), ipsilateral inflamed (46%, 5.87 to 8.58 nCi/mg) and contralateral inflamed (45%, 5.51 to 8.02 nCi/mg). The absolute magnitudes of these increases were 1.85, 2.71, and 2.51 nCi/mg, respectively (Fig. 15). Expressed in this way, the PI turnover stimulated by carbachol was greater after inflammation compared to non-inflamed by 46% (ipsilateral) and 36% (contralateral).

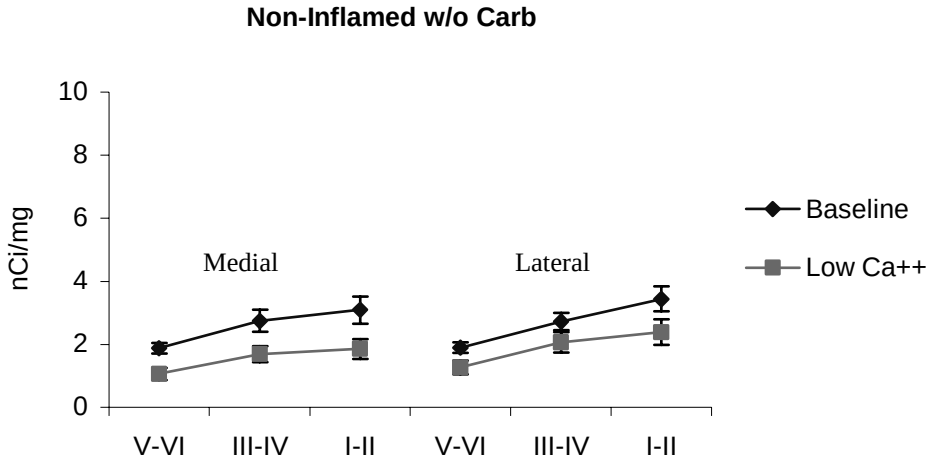

Figure 8. Effects of low Ca^{++} buffer on PI labeling in spinal cord sections from non-inflamed animals without carbachol stimulation. Low Ca^{++} buffer significantly reduced overall baseline turnover ($p<0.001$) (n=10). Data points represent average medians for medial or lateral region of each laminar group (+or- SEM).

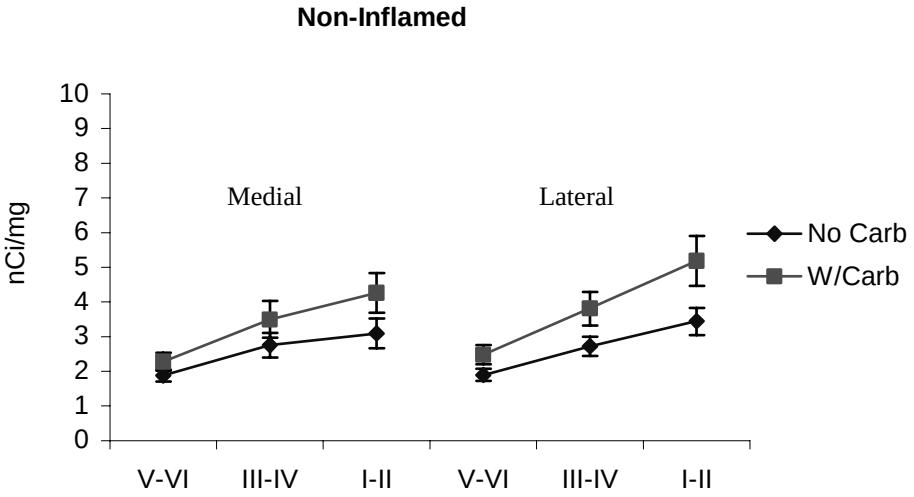

Figure 9. Effects of carbachol stimulation on PI labeling in spinal cord sections from non-inflamed animals. The addition of carbachol (n=5) to the incubation solution resulted in significantly higher levels of PI turnover in the dorsal horn compared to sections incubated without carbachol (n=10) ($p<0.001$). The largest increase was seen in the lateral half of laminae I-II. Data points represent average medians for medial or lateral region of each laminar group (+or- SEM).

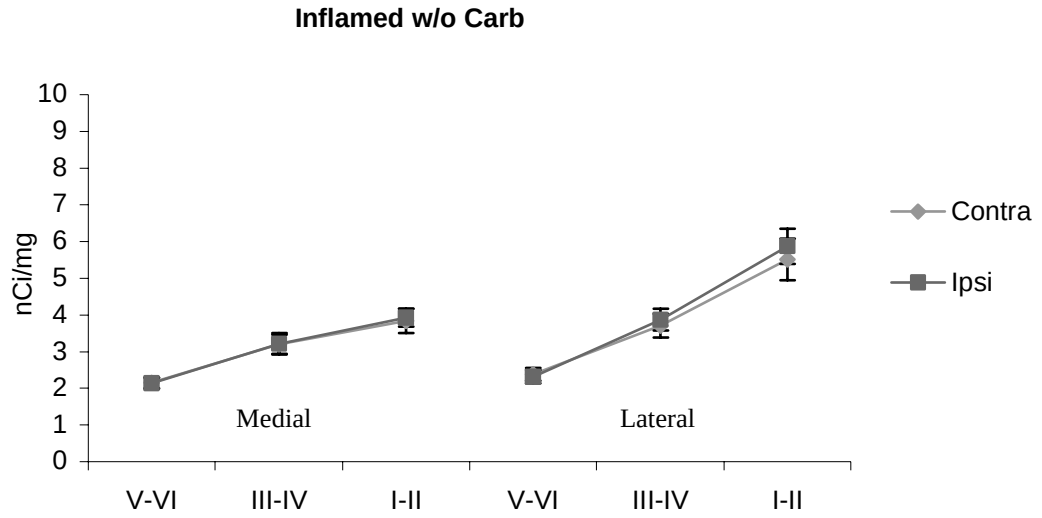

Figure 10. Effects of inflammation on baseline PI labeling. There was a significant effect of laminar location ($p<0.001$) ($n=10$). Post-hoc analyses revealed that labeling in the lateral half of laminae I-II was significantly higher than in other locations. Data points represent average medians for medial or lateral region of each laminar group (+or- SEM).

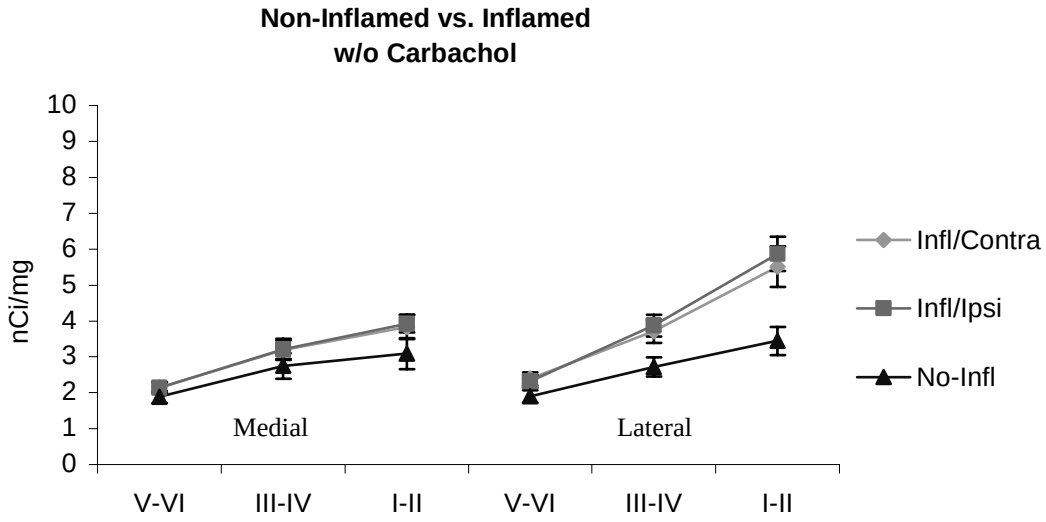

Figure 11. Comparison of baseline PI turnover between ipsilateral and contralateral sides of inflamed ($n=10$) animals to sections from non-inflamed ($n=10$) animals. Both ipsilateral and contralateral sides of inflamed animals were significantly higher than non-inflamed animals ($p<0.001$). Post-hoc analyses revealed significant increases in lateral I-II and lateral III-IV of both ipsilateral and contralateral sides over non-inflamed sections. Data points represent average medians for medial or lateral region of each laminar group (+or- SEM).

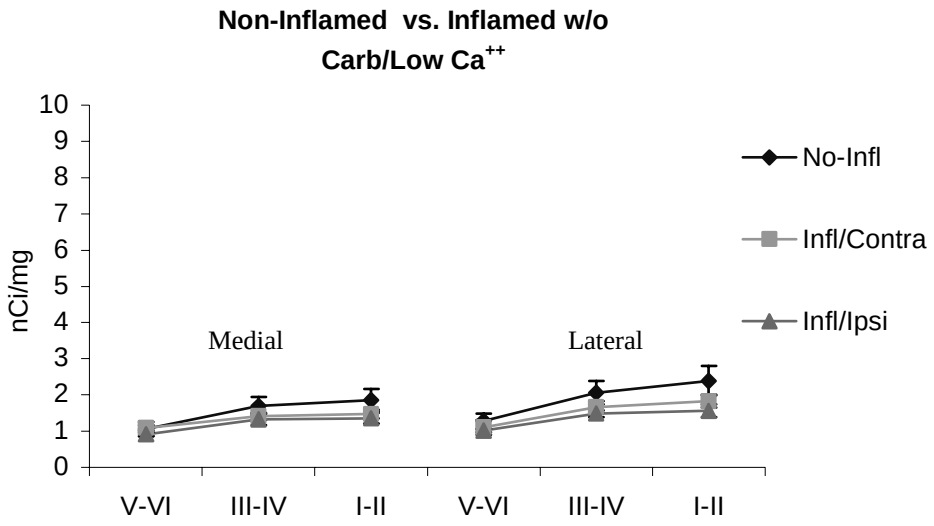

Figure 12. Effects of reduced synaptic activity (by low Ca^{++}) comparing ipsilateral and contralateral sides of inflamed animals (n=4) to sections from non-inflamed animals (n=4). Both ipsilateral and contralateral sides of sections from inflamed animal showed significant reductions in PI labeling when compared to non-inflamed animals ($p<0.001$). Data points represent average medians for medial or lateral region of each laminar group (+or- SEM).

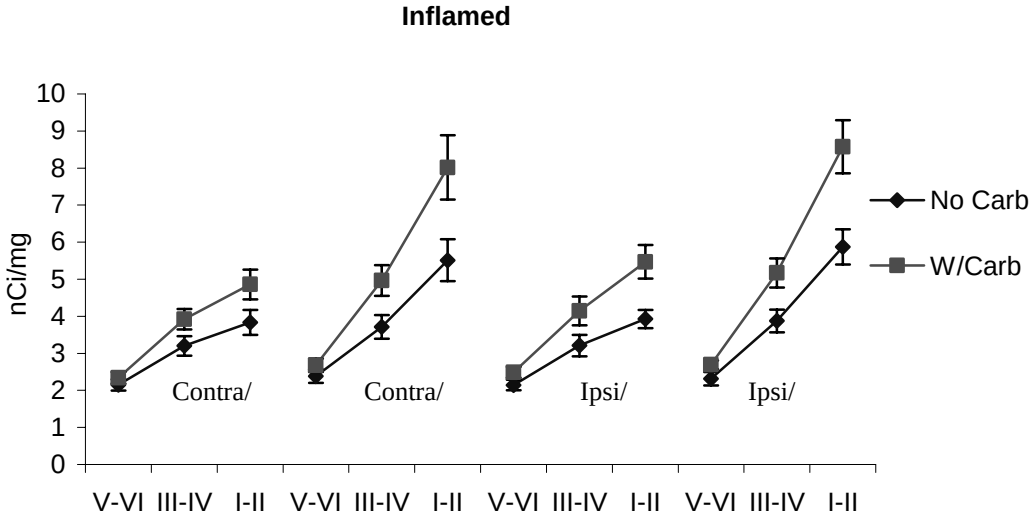

Figure 13. Effects of carbachol stimulation on sections from inflamed animal. A two analysis of variance revealed a significant interaction between treatment and laminar location ($p<0.006$). Post-hoc analyses demonstrated both the ipsilateral and contralateral sides, the lateral half of laminae I-IV and the medial half of laminae I-II showed significant increases. Data points represent average medians for the medial and lateral regions of the ipsilateral and contralateral sides (+or-SEM) (no carb n=10, w/carb n=8).

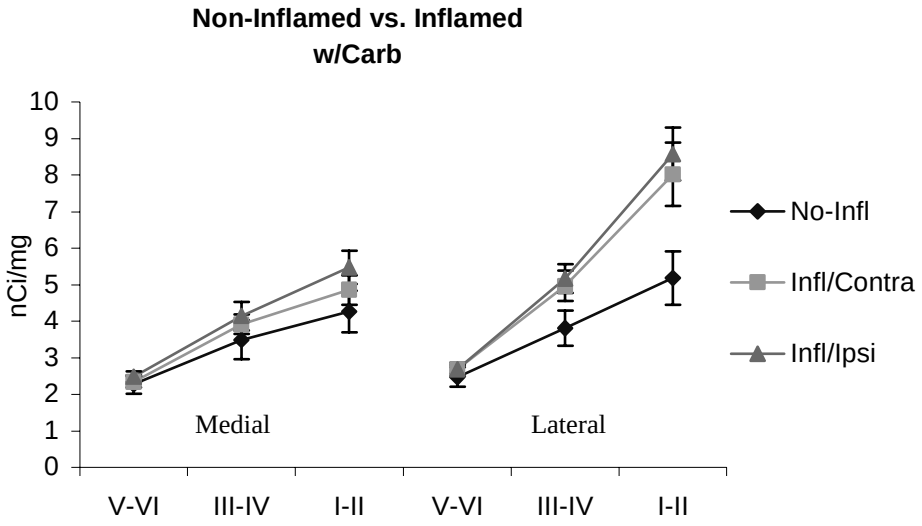

Figure 14. Effects of carbachol stimulation comparing the ipsilateral and contralateral regions of sections from inflamed animals (n=8) to sections from non-inflamed animals (n=5). Compared to sections from control animals, carbachol stimulated significantly higher levels of PI turnover in sections from inflamed animals ($p<0.001$). Post-hoc analyses revealed both the ipsilateral and contralateral sides of sections from inflamed animals were significantly higher with lateral I-II being higher than medial I-II. Data points represent average medians for medial or lateral region of each laminar group (+or-SEM).

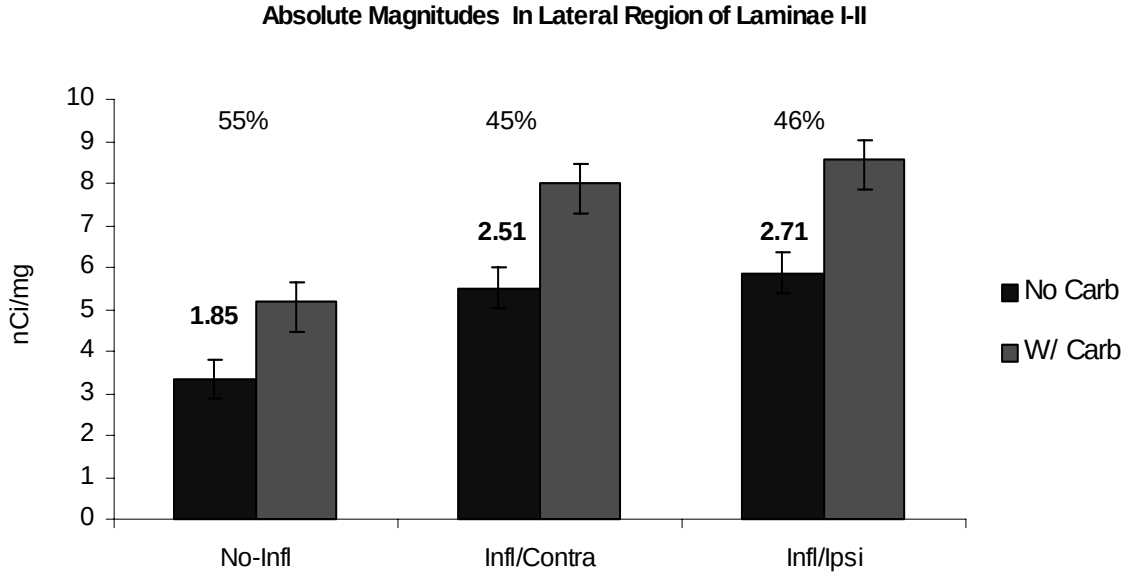

Figure 15. Demonstration of absolute magnitudes for carbachol stimulation in the lateral region of laminae I-II of sections from non-inflamed animals (n=5) and the ipsilateral and contralateral sides of sections from inflamed animals (n=8).

Chapter 4

Discussion

The present study utilized a technique that allows for the autoradiographic localization of PI turnover in the rat spinal cord. Measurement of PI turnover is usually done on cross-chopped slices (typically 350 x 350 µm). The slices are incubated with radiolabeled myo-inositol in the presence of lithium in Krebs buffer solution. Receptor activation resulting from the addition of a neurotransmitter agonist causes the accumulation of labeled inositol phosphates, which are analyzed by anion exchange chromatography. A major advantage of the present technique is that it allows for histological localization of PI turnover within the spinal cord. This is important in the study of pain because it allows for the comparison of PI turnover with nociceptive innervation of the dorsal horn. In addition it makes it possible to measure the magnitude and location of endogenous PI turnover. Typically in cross-chopped slice experiments PI hydrolysis is simply reported as a percent change over baseline (Prat et al., 1993; Parsons et al., 1995). Similar to the case of cross-chopped slice preparations the present study cannot determine how much PI turnover is due to PI linked receptors not accounted for by the agonist (agonist) or baseline turnover.

The present study demonstrated that superficial dorsal horn laminae expressed higher levels of PI turnover than deeper laminae across all treatment groups. This finding is consistent with previous work showing that muscarinic receptors (as well as other PI

linked receptors) are concentrated in lamina II followed by lamina III (Coggeshall and Carlton, 1997). An interesting finding was that the lateral region of the cord consistently expressed higher levels of PI labeling than the medial region. Mapping of the rat dorsal horn shows that the toes are situated medially with the plantar surface occupying a more lateral position (Snow and Wilson, 1991). This organization is consistent with the pattern of lateral PI labeling in the inflamed rats since the injection site was the plantar surface. This does not, however, explain the lateral labeling in the control animal. Although generally not commented on, there are several examples of lateralized effects in the superficial dorsal horn of the spinal cord. Results from Martin et al., (1999) suggests that CFA inflammation produces an increase in PKCγ in the lateral half of lamina IIi. Chronic sciatic constriction injury also appears to produce a distinct lateral increase in FOS immunoreactiviy (Yamazaki et al., 2001).

There is evidence for medial/lateral localization of receptors in the spinal cord. Results from Yang et al., (2001) suggest that GABAb immunoreactivity in the dorsal horn of L4 is laterally located. There is also very good evidence for SP receptors being medially located (Stucky et al., 1993; Woolf, 1987). However, work from Abbadie et al., (1996) shows that inflammation significantly increases SP immunoreactivity in the lateral region of the dorsal horn. They speculate that their lateral increase in SP immunoreactivity with inflammation is a result of medially located cell bodies whose dendrites arborize laterally. Consideration for future studies is the size and location of inflammatory injections. By utilizing smaller injection volumes one could test various

regions of the rostral-caudal aspect of the hindpaw with location of labeling in the dorsal horn.

A somewhat surprising finding was the increase in PI labeling in the contralateral dorsal horn following inflammation. The primary termination of afferent input is in the ipsilateral dorsal horn. One possible explanation is that unilateral inflammation increases the sensitivity of the contralateral side. This idea is supported by the work of Yamazaki et al., (2001) who found a significant increase in FOS immunoreactivity contralateral to sciatic nerve constriction. Also, chronic constriction injury to the infraorbital nerve increases sensitivity in the receptive field of the contralateral infraorbital nerve (Vos et al., 1994). Of further interest is the finding that inflammation results in a bilateral increase in 2-deoxyglucose (2-DG) in laminae I-II at two, four, and fourteen days post inflammation (Schadrack et al., 1999). In addition, differences with respect to side of cord were not seen until day fourteen. Since the 2-DG technique reflects regional glucose utilization, it is thought to be a measure of neuronal activity. The bilateral increase in PI turnover in the present study might be a reflection of this increased activity since 2-DG in the study of pain would reflect both nociceptive and anti-nociceptive activity. The bilateral increase in 2-DG labeling in laminae I-II was evenly distributed medial to lateral across the dorsal horn. In the rat, lamina I contains large numbers of terminals of primary afferents, propriospinal neurons, and interneurons (Chung et al., 1989). Primary afferents appear to be evenly distributed medial to lateral whereas propriospinal and interneuron terminals are located laterally in the dorsal horn (Coggeshall, 1981; Earle 1952; Szentagothai, 1964). It is generally thought that Ach-induced anti-nociceptive effects

arise from intrinsic neurons since evidence shows a lack of cholinergic projection neurons (Sherriff et al., 1991; Eisenach, 1999; Barber et al., 1984). Therefore the lateral effects found in the current study and by other researchers may reflect the activity of propriospinal and interneurons.

The origin of these contralateral effects is not clear. Willis and Coggeshall (1991) have reviewed the mapping studies of the dorsal horn, and from this work it appears that large axons from spinothalamic tract neurons in lamina deep to lamina I do not have collaterals. However, branching of small axons from lamina I was uncertain. Since the major input into lamina I arises from C and Aδ fibers the possibility exists that spinothalamic tract neurons originating from lamina I send collaterals to the contralateral dorsal horn while decussating in the cord. In addition, other studies have demonstrated that some primary afferent fibers send projections to the contralateral side (Culberson et al., 1979; Light and Perl, 1979; Sugiura et al., 1986).

A significant portion of baseline PI turnover in the dorsal horn for both inflamed and non-inflamed animals was synaptically mediated. Sections incubated in low Ca^{++} buffer showed a significant reduction in PI labeling in treatment groups. An unexpected finding was that low Ca^{++} buffer had a greater effect on sections from inflamed animals. Caution must be used in the interpretation of this finding since the numbers of animals in each group was small. A possible explanation for this comes from studies of a member of the regulator of G-protein signaling (RGS) family (RGS2) (Dohlman and Thorner, 1997; Koelle, 1997). RGS2 stimulates the GTPase activity of the α subunit of the G-protein G_q. Second messenger mediated receptors in response to an agonist undergo a conformational

change, which causes the Gα subunit to release GDP. Binding of GTP results in the dissociation of GTPα from the βγ subunits. In turn each of these components can regulate downstream effectors. The Gα subunit also has GTPase ability. This terminates the signal by converting GTP to GDP which allows for the reassociation of the α, β, and γ subunits (Siegel et al., 1999).

Ingi et al. (1998) report that RGS2 is rapidly upregulated in response to plasticity-inducing stimuli in brain neurons. Therefore increasing GTPase activity would decrease the duration of signaling by the α and βγ subunits. A possible explanation that low Ca^{++} buffer had a greater effect on sections from inflamed animals could be attributed to RGS stimulation of GTPase activity. If inflammation were sufficient to induce RGS increased GTPase activity then the duration of signaling by the subunits would be reduced. Therefore non-inflamed animals would express longer signaling by the subunit than inflamed animals. The addition of a low Ca^{++} buffer could have a greater effect on inflamed animals since their signaling duration would be shorter. This would mean that PI turnover in the presence of low Ca^{++} would be due to G-protein activation in the absence of an agonist. Several muscarinic receptors (including Gα) have been shown to activate G-proteins in the absence of an agonist (Costa and Herz, 1989; Tian et al., 1994; Samama et al., 1994; Barker et al., 1994; Leeb-Lunberg et al., 1994). This has been explained by allosteric models in which receptors exist in equilibrium between two conformations one of which interacts with agonists (Samama et al., 1993; Chidiac et al., 1994). Agonists are thought to increase the probability of G-protein interaction with the

active form. Even though less probable, G-proteins can interact with the active form in the absence of the agonist thereby stimulating a second messenger response.

To localize inflammation effects on PI turnover in this study, G-protein regulation, muscarinic receptor binding, and muscarinic receptor/G-protein coupling with inflammation need to be explored. Inflammation is known to increase affinity and immunoreactivity of other receptors in the dorsal horn associated with nociceptive input (Stucky, et al., 1993; Aanonsen et al., 1992; Abbadie et al., 1996; Kar et al., 1993; Schafer et al., 1993; McCarson and Krause, 1994). However, changes in receptor binding do not necessarily reflect the function of receptors. For example, increased expression of NK 1 receptors in cultured spinal cord neurons does not result in increased generation of PI (Abrahams et al., 1999). This is further supported by the work of Holland et al. (1993), showing that SP receptors and G proteins can uncouple with repeated stimulation. Recent evidence also indicates that changes in PI signaling can exist without changes in 5-HT receptor number or affinity (Toscano, 1999).

The results of this study provide further evidence for cholinergic involvement in pain processing and indicate a possible regulatory site within the anti-nociceptive pathway. Carbachol-induced increases in PI turnover with inflammation in this study most likely reflect upregulation of anti-nociceptive activity in response to the inflammation. This would represent an attempt to counteract the effects of increased nociceptive processing. There is considerable evidence to support the finding that ACh is anti-nociceptive in the dorsal horn. Spinal cord ACh content is significantly increased following formalin injection, and intrathecal injections of muscarinic antagonists inhibit

the second phase (prolonged phase of tonic pain) of the nociceptive response (Honda et al., 2000). Intrathecal administration of carbachol (a muscarinic agonist) or acetylcholinesterase inhibitors produces anti-nociception (Taylor et al., 1982; Gillberg et al., 1989; Abram and O'Connor, 1995; Bouaziz et al., 1995) that can be inhibited by the muscarinic antagonist atropine (Zhuo and Gebhart, 1991; Naguib and Yaksh, 1994). Cholinergic anti-nociception appears to involve both muscarinic and nicotinic receptors since both produce analgesia when administered intrathecally, and the analgesia can be reversed by addition of specific nicotinic and muscarinic antagonists (Yaksh et al., 1985; Rao et al., 1996; Lawand et al., 1999; Marubio et al., 1999; Pan et al., 1999). However, most of the anti-nociceptive effects of acetylcholine appear to be mediated through muscarinic receptors: intrathecal muscarinic antagonists nearly abolish anti-nociceptive effects whereas nicotinic antagonists only attenuate about 40% of the effect (Pan et al., 1999). The effects of nicotinic agonists are further complicated depending on their route of administration. If administered intrathecally they demonstrate nociceptive and anti-nociceptive properties (depending on concentration), whereas, if given systemically they produce anti-nociceptive effects (Rueter et al., 2000).

A possible future direction might be to study the time course of inflammation using measures of carbachol stimulated PI turnover. Since two days post CFA inflammation represents peak nociceptive effects (Honore, 1999) larger increases in anti-nociceptive effects may be seen at time intervals beyond two days. This could be why higher 2-DG labeling is seen after four days post inflammation, but not at one and two days post inflammation. It is possible that chronic pain may reflect a deficit in anti-

nociceptive processing in addition to upregulated nociceptive input. Follow-up studies should include inflammation effects on muscarinic receptors and G proteins, since both have a direct impact on PI turnover.

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